Histol Histopathol (2007) 22: 1205-1211

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Increase of annexin 1 immunoreactivity in spinal cord of newborn opossum (*Monodelphis domestica*) at the time when regeneration after injury stops being possible

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Summary. Annexins constitute a family of proteins that associate reversibly with cell membranes in a calcium dependent manner. We have studied the distribution of annexin 1, which is known to mediate anti-inflammatory actions of glucocorticoids, and which is upregulated after spinal cord injury, in newborn and adult South American opossum (*Monodelphis domestica*) spinal cord. We show the increase in the annexin 1 immunoreactivity in spinal cords of neonatal opossums over the critical period when regeneration after injury ceases to be possible. We further show the restricted and specific sites at which it is detected in adult opossum cerebellum and hippocampus. Since the procedures used in immunochemistry of annexin in CNS have in the past yielded conflicting results, different procedures were tested and shown to be reliable. As a control, annexin 1 distribution was surveyed in kidney.

Key words: Annexin 1, Opossum, Immunoreactivity, Spinal cord, Brain

Introduction

The annexin family of calcium-binding proteins consists of more than ten mammalian genes, characterized by a conserved core domain and a unique amino terminal region. Annexins bind (annex) to phospholipids in the presence of calcium. They have been implicated in numerous biological processes including: intracellular signaling, cellular adhesion, cellular trafficking, control of inflammation, mitosis and apoptosis (Raynal and Pollard, 1994). Their expression is highly localized in a characteristic manner in specific cells of various organs including the nervous system and the kidney (Fava et al., 1989; Dreier et al., 1998). As yet, their functions in the CNS are not known.

Here we focus on Annexin 1 (lipocortin 1, p35, calpactin II, chromobinding 9, GIF), the first member of the annexin superfamily of proteins to be characterized (Parente and Solito, 2004). Annexin 1 mediates anti-inflammatory actions of glucocorticoids (Wu et al., 1995), by controling the activity of cytosolic phospholipase A2. This in turn regulates biosynthesis of eicosanoids (prostaglandins, leukotriens). In addition to their role in inflammation, the eicosanoids and phospholipase A2 mediate growth cone repulsion and collapse (de la Houssaye et al., 1999; Mikule et al., 2003). Since annexin 1 is upregulated after spinal cord injury (Liu et al., 2004; Mladinic and Digby, unpublished) the possibility arises that it might play a part in preventing nerve fibers from regenerating after spinal cord injury. This idea is supported by the finding that annexin 1 is upregulated in: lesioned cerebellum (Young et al., 1999), patients with astrocytosis following CNS lesions (Johnson et al., 1989; Eberhard et al., 1994), patients with multiple sclerosis (Elderfield et al., 1992; Probst-Cousin et al., 2002), and animals with experimental allergic encephalomyelitis (Bolton et al., 1990).

A favorable preparation for testing the role of annexin 1 in repair processes of mammalian CNS is the opossum spinal cord. Neurons in immature spinal cord can regrow after injury so as to restore function. However, this permissive state stops abruptly at about 12 days of age (Nicholls and Saunders, 1996). Thus, complete transection of cervical spinal cord made at 9 days is followed by regeneration of injured axons, but after that repair becomes impossible. Here we investigate annexin 1 immunoreactivity in the opossum spinal cord over the transition period that is critical for regeneration.

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In addition we surveyed the distribution of annexin 1 in adult spinal cord and brain. For this we tested a variety of fixation and immuno-procedures. This was necessary because in previous studies of annexin 1 in CNS, discrepancies arose in the sites of distribution that were reported, probably as a result of difficulties in fixing CNS annexin 1 reliably for histochemistry. A useful control for our procedures was that in rat, human and rabbit kidney, annexin 1 immunoreactivity is clear and reliable (Fava et al., 1989; McKanna et al., 1992; Dreier et al., 1998; Tribolo et al., 2000).

We show here the immunoreactivity of annexin 1 in adult opossum spinal cord, cerebellum and hippocampus. Moreover, we show that the increase in annexin 1 immunoreactivity occurs in the young spinal cord at the critical time at which ability for regeneration becomes lost.

Materials and mehtods

Dissection of opossum CNS

Pups from opossums (*Monodelphis Domestica*), bred and maintained as described previously (Nicholls et al., 1990), were removed from their mothers 6 or 14 days after birth, anesthetized with metofane and killed by rapid excision of the heart and lungs. The entire central nervous system was dissected in oxygenated Basal Medium Eagles (BME, Gibco), embedded in Tissue-Tek O.C.T. embedding compound (Sakura Finetek), frozen on dry ice and stored at -80°C until use. Anesthetized adult and 2 month old opossums were killed and their CNS and kidneys were dissected as described. 3-5 animals of each age were used for these experiments.

Immunofluorescence

12-14 µm cryostat sections of the kidney, brain or cervical spinal cord were cut, thaw-mounted onto SuperFrost Plus glass slides (Menzel-Gläser) and air dried. After 20 min fixation in 4% paraformaldehyde in Phosphate buffered saline (PBS), slices were washed for 30 min in PBS, dehydrated 2 min in 100% ethanol and stored at -20°C. The primary polyclonal antibodies used were: Rabbit anti-Annexin I (Zymed Laboratories) or Goat anti-Annexin I (N-19): sc-1923 (Santa Cruz Biotechnology). These antibodies were shown previously to specifically react with human or rat annexin 1, respectively (Garcia Pedrero et al., 2004; Liu et al., 2004). After washing in Tris buffered saline (TBS), slices were permeabilized 2 min on ice in 0.1%Triton X-100/0.1% sodium citrate and washed three times in TBS, 5 min each. Slices were incubated 30 min in 10% Bovine serum albumine (BSA, Sigma) in TBP at room temperature, followed by 12-24 h incubation in TBS containing 50 times diluted primary antibody, 1% BSA and 1% secondary antibody species specific serum. After the three washes in TBS, 5 min each, the slices were incubated in 50 times diluted secondary antibody (Bovine anti-rabbit IgG-R: sc-2367 or Chicken anti-goat IgG-FITC: sc-2988, Santa Cruz Biotechnology) in TBS. After washing in TBS (3x5 min) sections were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) fluorescent dye and mounted in Vectashield mounting medium (Vector laboratories). Control sections were processed as described above, except that the primary antibody was omitted. The controls for each antibody never exhibited immunoreactivity. For each experiment 10-20 tissue sections, from 3-5 different animals were analyzed.

Immunohistochemistry

Immunohistochemistry was performed on the tissue sections, fixed and cut in the same way as for immunofluorescence. In addition, the fresh tissue was fixed for 1 hour at room temperature in 5.5% acrolein, prepared in 0.1M acetate buffer. After fixation, tissue was cryoprotected by incubation in sucrose solutions (increasing concentrations from 10% to 30%), embedded in Tissue-Tek O.C.T. embedding compound (Sakura Finetek) and frozen on dry ice. 10 µm cryostat sections were cut, thaw-mounted on SuperFrost Plus glass slides (Menzel-Gläser) and stored at -20°C until use.

The tissue was processed as for immunofluorescence until secondary antibody incubation, but using Phosphate buffered saline (PBS) instead of TBS. Then, the Vector Laboratories Vectastain ABC system with DAB as the chromagen (Vector Laboratories) was used according to the manufacturer's protocol. At the end, the tissue was dehydrated and cleared in ethanol and xylene and mounted using EUKITT (Kindler) mounting medium. As before, in each experiment 10-20 tissue sections from 3-5 different animals were used.

Results

Several different immunostaining protocols were used to analyze annexin 1 immunoreactivity in adult and immature opossum CNS. The procedures included the use of paraformaldehyde (PFA) and acrolein fixation, two different annexin 1 specific antibodies and different fluorescent and non-fluorescent secondary antibodies. We did not observe differences between the annexin 1 immunoreactive signals obtained using different immunostaining protocols on tissue fixed with PFA or acrolein.

We first tested the reliability of our fixation and immunostaining protocols in opossum by analyzing its immunoreactivity in kidney, since its localized distribution there in other animals has been well established (Fava et al., 1989; McKanna et al., 1992; Dreier et al., 1998; Tribolo et al., 2000). We found annexin 1 to be specifically localized in the cortical glomeruli and medullary/papillary collecting ducts of the adult opossum kidney (Fig. 1). These correspond to sites of annexin 1 immunoreactivity in rabbit kidney: epithelial cells of Bowman's capsule, membranes of

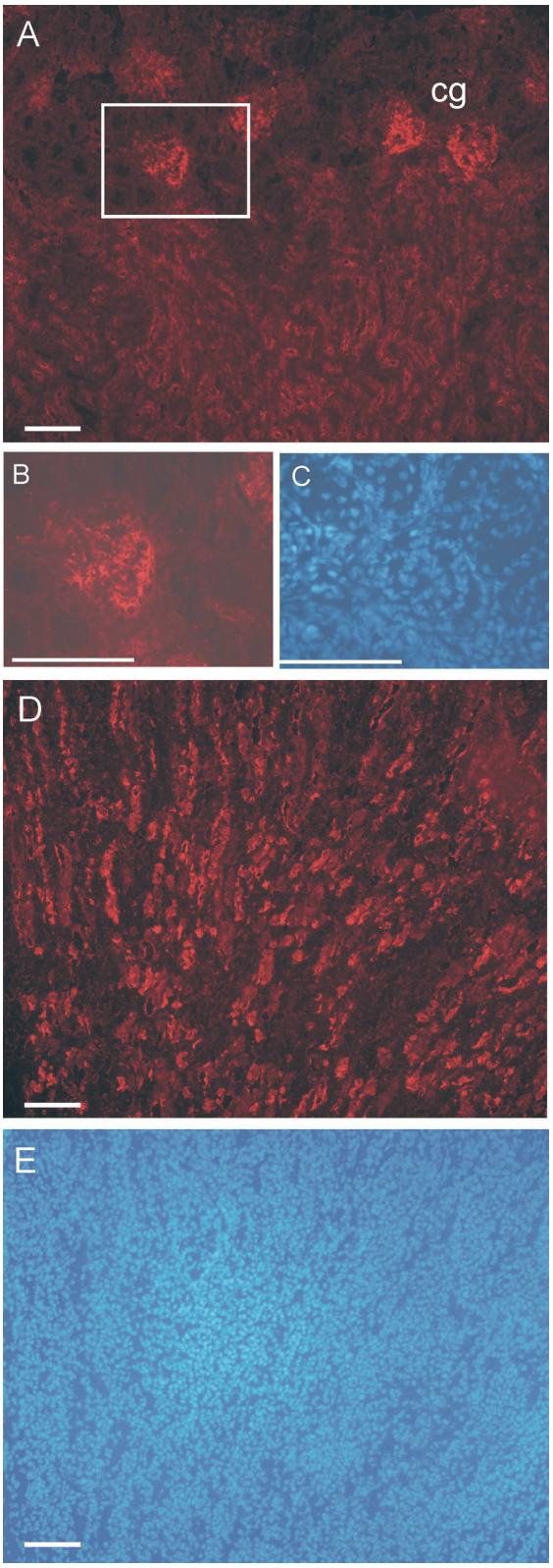

Fig. 1. Annexin 1 in adult opossum kidney. Annexin 1 immunostaining is present in glomerular cells of the kidney cortical glomeruli (cg) (**A** and higher magnification in **B**) and in the medullary/papillary collecting ducts (**D**). In **C** and **E** the DAPI counterstaining of the same regions as in **B** and **D** is shown. Scale bars: 10 µm.

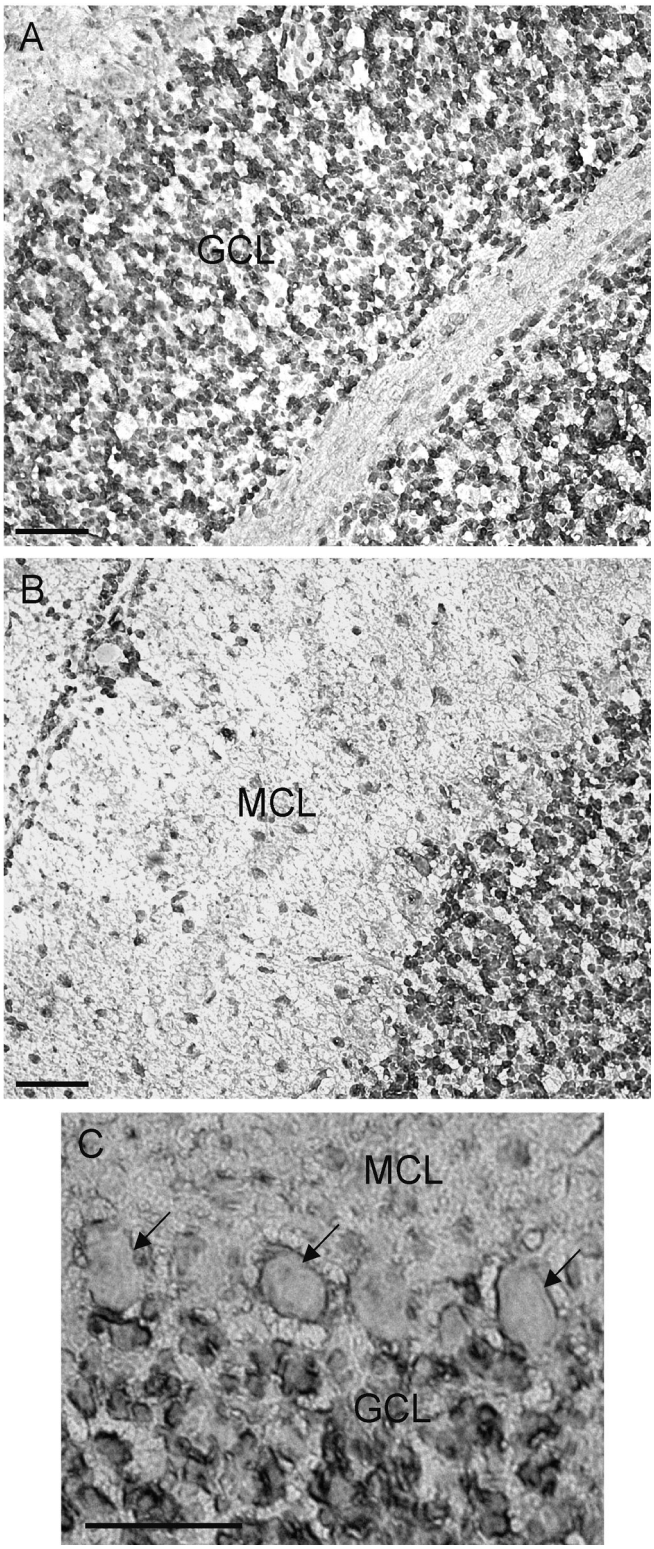

Fig. 2. Annexin 1 in saggital sections of adult opossum cerebelar cortex. The signal is prominent in the GCL (**A**). The scattered cells are positive in the MCL (**B**), while Purknije cells are avoided from signal (indicated with arrows in **C**). The perinuclear staining can be observed in the cerebellar granule cells in the GCL (**C**). GCL: granule cell layer, MCL: molecular cell layer, Arrows: indicate Purkinje cells. Scale bars: 10 µm.

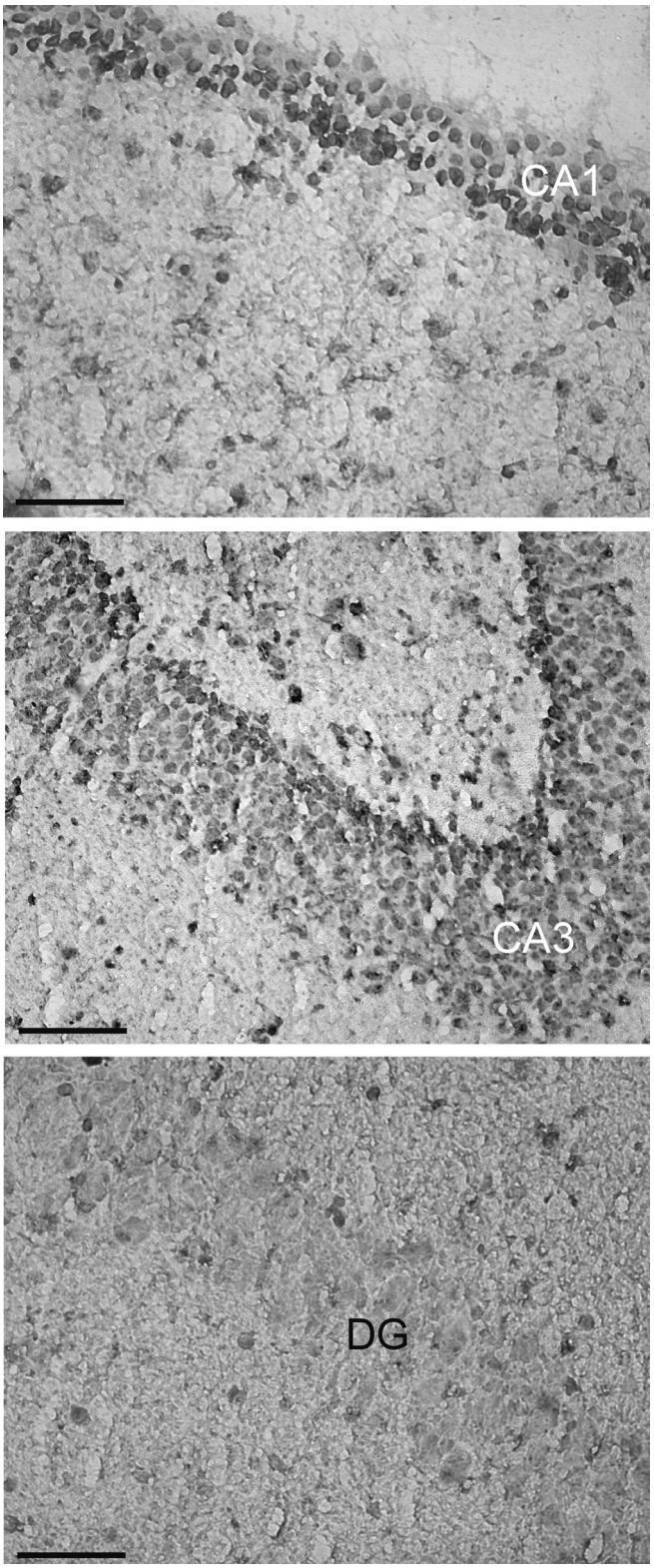

Fig. 3. Annexin 1 in adult opossum hippocampus. In saggital sections of the adult opossum hippocampus annexin 1 immunoreactivity is observed in CA1 (**A**) and CA3 region (**B**), while the signal is absent from the dentate gyrus (DG, in **C**) neuronal cells. Scale bars: 10 mm.

collecting duct cells, and restricted localization in principal and glomerular cells (Tribolo et al., 2000).

The annexin 1 immunoreactivity was localized in specific regions of opossum brain and spinal cord. In adults, annexin 1 specific immunostaining was localized to cerebellum (Fig. 2) and hippocampus (Fig. 3), with only a few labeled cells in medulla oblongata (data not shown). In the cerebellum prominent annexin 1 perinuclear staining was observed in the granule cells (Fig. 2A) and in cells in the molecular layer (Fig. 2B). Purkinje cells did not show any annexin 1 labeling (Fig. 2C). In the hippocampus, annexin 1 positive neurons were found in the Ammon's horn, of which CA1 and CA3 regions are shown in Figs 3 A,B. Dentate gyrus neurons showed no annexin 1 labeling (Fig. 3C).

In Fig. 4 we show that an increase of the annexin 1 immunostaining occurs in the cervical spinal cord during

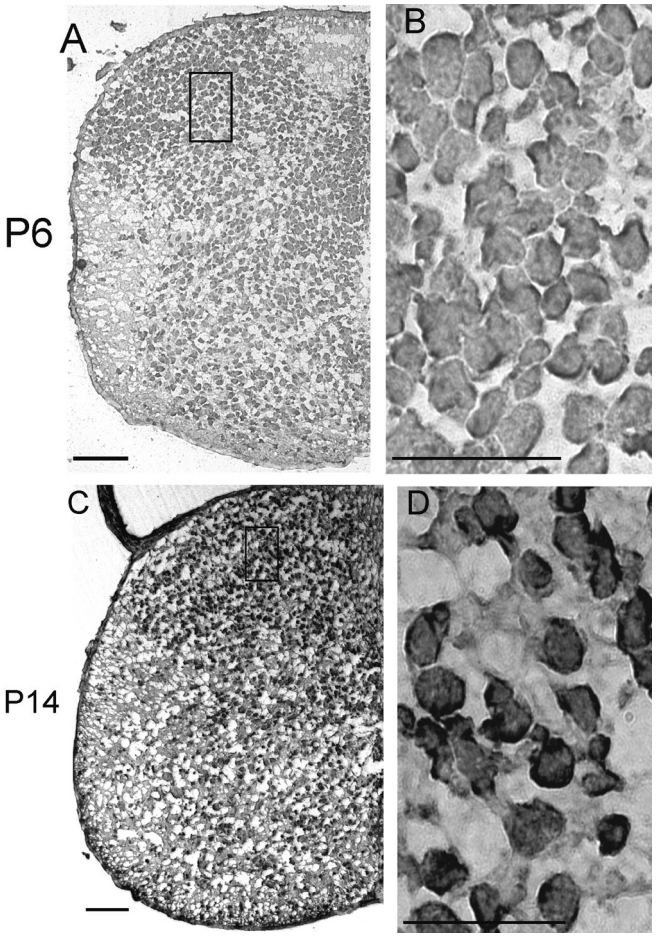

Fig. 4. Annexin 1 in opossum cervical spinal cord at times when regeneration stops being possible. The annexin 1 immunoreactivity is shown on transverse sections of the opossum cervical spinal cord (SC) at postnatal day 6 (P6) when the spinal cord can regenerate after injury (**A** and higher magnification of the part of the dorsal horn in **B**) and at postnatal day 14 (P14) when it can no longer regenerate (**C** and higher magnification of the part of the dorsal horn in **D**). Scale bars: 100 μm.

the second week of postnatal development, at the time when regeneration of the spinal cord stops being possible. At postnatal day 6 (P6), when the regeneration of the spinal cord after injury is possible, annexin 1 shows week immunostaining in the dorsal horn. At the P14, when regeneration after injury is no longer possible, there is pronounced annexin 1 immunostaining, mostly in the dorsal horn and dorsal root, with scattered positive cells also in the ventral horn. Motoneurons did not show any annexin 1 labeling.

In adult spinal cord the annexin 1 immunoreactivity disappeared, except for the ependymal cells lining the central canal (Fig. 5).

Discussion

The annexin 1 immunoreactivity was investigated in the neonatal opossum spinal cord at the time when regeneration of the spinal cord stops being possible, and in the adult. The highly localized distribution of the annexin 1 in opossum kidney served as a positive control for our experimental procedures, used subsequently to demonstrate its distribution in CNS. The results show that annexin 1 immunoreactivity is increased in the neonatal spinal cord at the very time when it loses its ability to regenerate after injury. Later, in the adult, neurons and glial cells fail to be labeled in the spinal

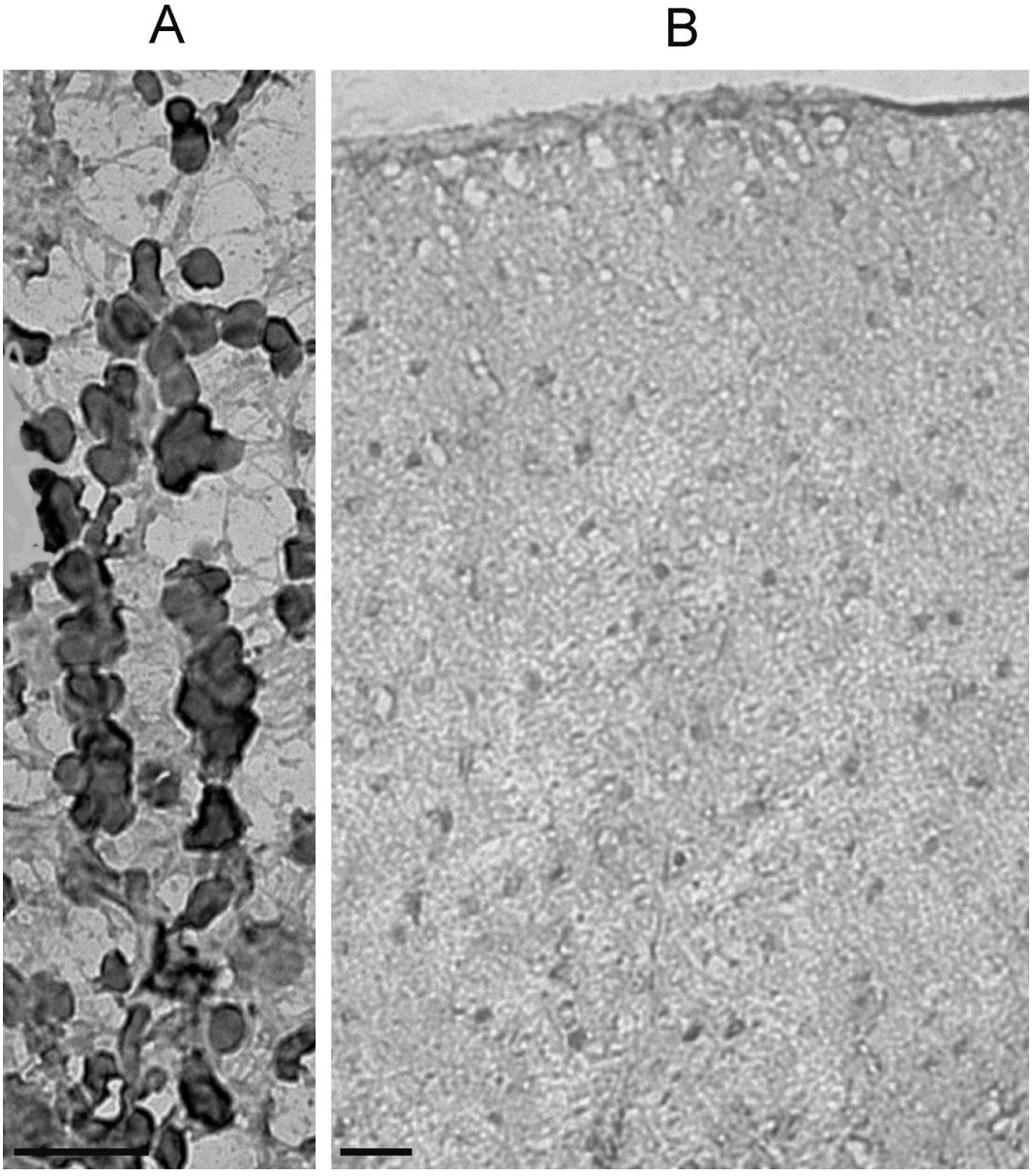

Fig. 5. Annexin 1 in adult opossum spinal cord. In the transverse sections of the opossum adult spinal cord the ependymal cells lining the central canal of the spinal cord show annexin 1 immunoreactivity **(A)** in contrast to neurons or glial cells **(B)**. Scale bars: 100 μm

cord.

Conflicting results have been reported about the immunolocalization of annexin 1 in the CNS during development and in pathophysiological conditions. These were believed to result in large part from unusual properties of the protein and its apparent sensitivity to different fixation conditions. Thus, the annexin 1 was detected in neurons and reactive astrocytes in one series of papers in which formalin, paraformaldehyde, Carnoy's fluid, ethyl alcohol and glacial acetic acid were used as fixatives (Johnson et al., 1989; Strijbos et al., 1991; Elderfield et al., 1993; Eberhard et al., 1994; Voermans et al., 1996; Huitinga et al., 1998). In contrast, other studies with formalin-acetate fixative detected annexin 1 only in microglia (McKanna, 1993; McKanna and Zhang, 1997), while others, using methanol, formalin or paraformaldehyde fixation detected annexin 1 in neurons, glia, and microglia (Dreier et al., 1998; Young et al., 1999; Liu et al., 2004). At least part of this variability could be accounted for by the sensitivity of annexin 1 immunoreactivity to pH, freezing and dehydration (McKanna and Zhang, 1997).

In our study we observed no differences between the annexin 1 immunoreactive signals in kidney or CNS when we used different immunostaining protocols on tissue fixed with PFA or acrolein. However, in contrast with the other studies where tissue was usually PFA-fixed before sectioning, we immediately froze and sectioned the freshly dissected tissue. Only after this was the tissue fixed briefly in PFA. Although this procedure can produce tissue damage, it provides the molecular integrity of proteins in tissue.

In the adult opossum brain annexin 1 was detected only in specific regions of the hippocampus and cerebellum. In the hippocampus annexin 1 immunostaining was present in the neurons of Ammon's horn (CA1, CA2 and CA3 regions), while no signal was detected in the dentate gyrus. In the cerebellum it was present in the granular layer but absent from the Purkinje neurons. In the adult spinal cord the annexin 1 immunoreactivity is reduced to the ependymal cells lining the central canal of the spinal cord. Those cells may retain the proliferative properties of neural stem cells (Fu et al., 2003). Dreier et al. (1998) have emphasized the annexin 1 expression in epithelial cells that line the lumen of different organs. At all sites, staining was perinuclear, and cytoplasmic, which is in accord with the fact that no nuclear localization sequences have been found in the primary structure of annexin proteins.

Our findings of the low level and localized signal of annexin 1 in adult opossum brain, confirms earlier studies of annexin 1 immunoreactivity. Thus, in rats, as in adult opossums, annexin 1 was seen in ependymal cells, subependymal astrocytes, meninges and neurons, but not in white matter (Probst-Cousin et al., 2002). In the hippocampus annexin 1 immunoreactivity was found in neuronal cell bodies of CA1, CA2 and CA3 and to a lesser degree in the granular cells of the dentate gyrus (Strijbos et al., 1991; Eberhard et al., 1994). In rat cerebellum, different distributions of annexin 1 immunoreactivity were reported by Mullens et al. (1994) - barely detectable, and Young et al. (1999) - expressed in molecular layer interneurons and Purkinje cell bodies.

However, what earlier reports do agree about is the upregulation of annexin 1 in pathological conditions and after lesions in CNS (cited in Introduction). Thus, annexin 1 upregulation was observed in reactive astrocytes and microglia in rat cerebellum after kainite lesions (Mullens et al., 1994; Young et al., 1999), and in neurons of human brains after injury or neurodegenerative diseases (Eberhard et al., 1994). In the adult rat spinal cord after traumatic injury, the annexins, including annexin 1, increased both in neurons and glial cells.

Our finding that annexin 1 is increased in neurons of the neonatal spinal cord at the time it loses the capacity to regenerate after injury suggests that it could play a role in molecular changes that are responsible for the transformation of the regenerative into non-regenerative CNS. In addition, the expression of annexin 1 in ependymal cells of the adult spinal cord, which may retain proliferative properties as neural stem cells, raises the possibility that annexin 1 may play a role in the plasticity of developing and adult mammalian CNS.

Acknowledgements. This work was partially supported by a grant from FVG. Miranda Mladinic is a postdoctoral fellow of Fundazione Kathleen Foreman Casali. We wish to thank Dr Marco Stebel and Mr Salvatore Guarino for their excellent work in maintaining our opossum colony, to Dr Gabriella Rossi, Dr Federica Ferrero and Mrs Jessica Franzot for their excellent technical help and to Dr Sergio Graziosi for his unfailing help with computers.

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Accepted May 9, 2007